DEFENSE ADVANCED RESEARCH PROJECTS AGENCY

R & D STATUS REPORT

ARPA ORDER NO.
#4507

PROGRAM CODE NO.
#N00019

CONTRACT NO.
N00014-82-K-0680

CONTRACT AMOUNT
$438,905.00

SHORT TITLE OF WORK

ULTRASENSITIVE DETECTION OF CHEMICAL SUBSTANCES

REPORTING PERIOD
JANUARY 1, 1983 - MARCH 30, 1983

EFFECTIVE DATE OF THE CONTRACT
JULY 1 1982

EXPIRATION DATE OF THE CONTRACT
JUNE 30, 1983

PRINCIPAL INVESTIGATOR
ROGER G. DEAN, Ph.D.

UNIVERSITY OF NEW MEXICO

SCHOOL OF MEDICINE

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I. PROGRESS REPORT PERIOD FOR JANUARY 1, 1983 TO MARCH 30, 1983

Studies are being conducted on

In the third quarter of our contract we have made great progress. Most of the progress has been in the area of hybridoma and monoclonal antibody development. We have, also, completed most of the task areas outlined in our original proposal for the first year. The following is a summary of the work done in the task areas.

Work is summarized for the following tasks:

TASKS

1. Preparation/purification of hapten carrier:

In the very early months of our research we found that the acetylcholinesterase from Sigma Chemical Company could be used without purification or modification ofr conjugation to DFP, binding to solid supports, or immunization of mice and rabbits. In the future we may electrophoretically purify or modify the Sigma product or produce our own acetylcholinesterase, however, these measures are not necessary at this time.

2. Formation of hapten carrier adducts:

AChE-DFP adducts were first made in the 5th month of the contract. The method now used is a modification of the method of Lanks and Seleznick (W. Lanks and M. Seleznick, Brochemica et Biophysica Acta 660 (1981) 91-95). Briefly, AChE is dissolved in .01M mops buffer at pH 7.6 incubated at 4° with DFP at a concentration of $5x10^{4}$M/ugn of enzyme for 24 hours. At the end of incubation the solution is dialyzed and concentrated in a micro-pro di con dialyzer. Protein recovery is between 45 and 53%. Enzyme inhibition is quite complete with better than 98% inhibition.

3. Production of hybridoma cell lines: → p. 2

Mock hybridization of NS 1 cells to mouse spleens was begun in November together with AChE-DFP immunization of mouse spleens in vitro. Immunization in vivo with AChE-DFP was begun in January 1983 during the past month we have been particularly successful at producing hybridomas. The procedures now used for immunization with AChE-DFP are as follows:

In Vitro:

10^{7} Naive mouse splenocytes are cultured in a 1 ml volume in 33 mm petri dishes in the presence of 10 ug of antigen for a 3-4 day period in the following conditions:

Medium: Complete Iscoves + 20% FCS.
Agitation: On a rotating table at 5 rpm.
Supplementation: Addition of nutritional mixture + FCS daily.
Temp/CO_2: 37° C/6%.

In Vivo:

Mice are immunized by IP injection of AChE-DFP either in Freund's adjuvant or precipitated with alum. The immunization schedule comprised three or more exposures of antigen followed by harvest of splenocytes for fusion three days following the last exposure.

Briefly, the fusion protocol now used is to expose NS 1 cells and primed mouse splenocytes (BALB/C) at a 1:2 to 1:4 ratio in 1 ml of PEG 1500 pH 8.0 for 3 min. during which the cells are resuspended and then pelleted by centrifugation. The supernatant is then discarded and the remaining PEG is diluted with 5 mls of medium over a 2 min. period. After two minutes the cells are resuspended and then repelleted by centrifugation. The cells are finally resuspended in complete Iscoves/20% FCS to yield 10^7 total cells/ml and then plated at 10^6 cells/well to date three sets of splenocytes have been immunized in vitro and hybridized with NS 1 myeloma cells yielding 54 clones surviving HAT selection. Two mice have been immunized and carried through the stage of fusion yielding 96 clones. We can anticipate at full production the immunization of six to eight spleens (both in vitro and in vivo) per month giving approximately 180-250 clones to be tested.

4. Production selection and characterization of monoclonal antibodies with specificity for trapped target molecules:

As of April 1 we had approximately 150 microtiter wells containing clones which were screened for the production of mouse immunoglobulin using the BBL streptavidin assay. Of these using a baseline cut off selection method we estimate 15-20% of these clones to be positive for mouse immunogloblin. In subsequent testing of 90 of these microtiter plate wells fifteen of these clones demonstrated antibody against AChE-DFP both in latex agglutination and nitro cellulose spot test. Tests are continuing toward the isolation of specific monoclonal antibody against AChE-DFP conjugate.

→p.3

5. Fabrication of solid supports with bound trapping moiety. The trapping agent AChE has been bound successfully to untreated polystyrene by passive hydrophobic bonding. The procedure followed for the polystyrene plates is to dilute AChE into carbonate buffer pH 9.46 to .125 units per 100 ul. 100 ul is added to each well and incubated overnight at 4° in a moisture box. After incubation the supernatant is removed and the well surface to which the AChE is bound, is washed with PBS. Plate wells coated in this way demonstrate good activity up to about 24 hours after coating at which time the activity is diminished. Polystyrene is known to inactivate enzymes and, therefore, may not be ideally suited to long term binding of acetycholinesterase. Polystyrene is, however, useful as a solid support in the monoclonal selection assays. Polystyrene supports will, also, be useful in initial testing of an ultrasensitive binding surface when testing can be accomplished within a period of about a day.

Several other surfaces have been tested for their usefulness as a

solid support in ultrasensitive assays. The criteria for judging is that they have little or no background noise in terms of non specifically bound fluorescent signal packets and they are hydrophilic enough to prevent dematuration of AChE. Polycarbonate, polyacrylamide, plexiglass, nylon 66 film, paraformaldehyde and mylar (terpthalate polyester) all show high backgrounds and are quite hydrophobic. To date the most promising solid supports for long term ultrasensitive detection appear to be a product by 3M which is agarose coated mylar and matt mylar which is a mylar with a frosted surface. These surfaces together with other products which are yet to be tested will be intensively studied in the second and third year of our contract.

6. Production of fluorescent loaded antibody signal packets;

We have chosen for our initial evaluation of signal packets, small (0.5-1 um diameter) fluorescent microspheres provided by Covelent Technology Corporation. The spheres that we are using at this time are designated MX and bind to primary amine groups of any protein. A wide variety of beads with various binding affinities are available. Two critical factors are important in selecting signal packets. First, when the beads are interacted with a solid support surface which is coupled to a protein trapping agent there should be little or no non specific binding. Secondly, the beads must have the proper characteristics such that when they are specifically bound they are not removed by the washing procedures.

Our research thus far indicates that the covaspheres satisfy both of these requirements when proper measures are taken to eliminate noise. In addition, covaspheres are available in a variety of surfaces which can be easily bound to proteins. Two surfaces have been found that have low noise characteristics with regard to covaspheres. The best surfaces are matt mylar and agarose coated mylar which give non specific binding counts estimated at less than 8 beads per cm^2. This level is achieved on a surface untreated with blocking protein and washed with phosphate buffered saline rather than dissociating agents. Thus, improvements can be made with these surfaces with relative ease. At present we are using the polystyrene surface to begin work on binding covaspheres specifically to the surface through a high affinity linkage. While the polystyrene surface does have a high non specific binding 600 spheres/cm^2 the system can be used to test for specific bonding and stability of bonds to washing procedures.

7. Initial evaluation of concept feasibility

To evaluate our approach to ultrasensitive detection several elements must be considered. The most important element in our scheme of detection is the ability to produce monoclonal antibodies, particularly to the determinate specific for the complex between the hapten and trapping agent. We have been quite successful in producing monoclonal antibodies. In the last month we have isolated over 90 hybridomas producing mouse immunoglobul with at least 15 of these clones active against AChE-DFP conjugate. We do not know yet if one of these clones is active against the conjugate determinant, however, success in this is determined in large part by the efficiency with which hybridomas with specificity toward the antigen can be produced. Considering the rate at which we now produce monoclonals it is reasonable to expect success by July 1, 1983. Of course, the effort

to isolate monoclonals will continue through the second year of the contract so that several monoclonals specific for the AChE-DFP conjugate may be isolated

Another important element in developing an ultrasensitive assay is to produce signal packets with appropriate fluorescent signal characteristics and the ability to find antibody. In addition, characteristics of low non specific binding to solid substrates and the ability to link to a solid substrate with specific binding that is stable to various washing procedures are important considerations in selecting signal packets.

We were very fortunate that Covaspheres, a new product of the Covelant Technology Corporation were being developed shortly before we began work. These beads have a very strong fluorescent signal and very little fading which gives excellent counting characteristics with a microscope at only 100 magnification. In addition, these spheres can be obtained with a variety of activated surfaces that bind antibody covelently. Using these beads we were able to demonstrate their feasibility as signal packets. The availability of these beads has enabled us to do some preliminary investigation of low noise factors and specific binding to solid substrates which is scheduled to be done in the second year of our contract. All early indications are that Covaspheres will work well in an ultrasensitive assay. While many other factors contribute to the success of our project the ability to make the appropriate antibody and utilize fluorescent signal packets are the most crucial. Our success with the Covaspheres together with our ability to produce monoclonal antibodies against the AChE-DFP antigen certainly indicate that our approach to ultrasensitive detection is feasible.

II. CHANGE IN KEY PERSONNEL

Because of the University's budget difficulties Dr. Dean's percent contribution to this project as well as his reimbursement was increased from 60% to 90% to more accurately reflect his actual contribution.

III. FISCAL STATUS REPORT

Please see enclosed report.

FISCAL STATUS

QUARTERLY REPORT

3RD QUARTER-JANUARY 1, 1983-MARCH 31, 1983

CATEGORY	BUDGET	1ST QUARTERLY TOTAL	2ND QUARTERLY TOTAL	3RD QUARTERLY TOTAL	QUARTERLY M-T-D JANUARY	QUARTERLY M-T-D FEBRUARY	QUARTERLY M-T-D MARCH	TOTAL ENCUMBRANCES	YEAR TO DATE TOTAL	FUNDS REQUIRED TO COMPLETE WORK (BUDGET BALANCE)
LETTER OF CREDIT	$438,905.00	91,925.11	82,003.17	24,186.91	40,842.82CR	22,836.72CR	22,683.20CR		198,115.19	
SALARIES-TECHNICAL	43,833.00				1,062.50CR					
SALARIES-PROFESSIONAL	57,248.00				10,908.23	9,093.24	9,093.24			
MATERIALS & SERVICES	24,171.00				2,045.37	2,298.17	4,785.84	5,438.63		
ANIMALS	5,871.00				95.93	89.47	326.41	412.51		
EQUIPMENT	135,476.00				3,524.67	3,149.00	390.00	800.00		
TRAVEL	30,000.00						636.00			
FRINGE BENEFITS	15,163.00				1,569.86	1,467.23	1,467.22			
CONSULTANTS	30,000.00					.01CR	.01			
OTHER	13,590.00				562.46	678.39	77.17	403.91		
INDIRECT COSTS	83,553.00				7,352.21	5,586.86	6,718.21			
TOTAL		91,925.11	82,003.17	24,186.91	15,846.59	474.37	810.90	7,055.05		

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		1,062.50CR					
		10,908.23	9,093.24	9,093.24			
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		3,524.67	3,149.00	390.00	800.00		
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		1,569.86	1,467.23	1,467.22			
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